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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No.: 10/666,642)
In re application of: JIANG, Cai-Zhong, *et al.*)
Filed: September 18, 2003)
Art Unit: 1638)
Examiner: BAUM, Stuart F.)
Docket No. MBI-0054)
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_____)

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 CFR 1.132 OF PETER REPETTI

I, Peter Repetti, declare:

1. I received my Bachelor of Science degree in Plant Science from The Pennsylvania State University and my doctoral degree in Plant Biology from The University of California, Berkeley. I joined Mendel Biotechnology in June of 2002 and have served as Senior Scientist since January of 2004. In this declaration, I serve as expert witness in that my work has involved characterization and use of cloned plant genes for modifying a variety of traits in genetically transformed plants, specifically in the areas of developmental alterations and the regulation of environmental stress responses. I have contributed to and supervised research in the area of environmental stress tolerance of plants that ectopically express sequences of the present invention, and I am therefore familiar with the present invention.
2. I understand that this application relates to transgenic plants transformed with an expression vector encoding a polypeptide having a conserved domain that is 70% identical to the conserved domain of G1274, SEQ ID NO: 194, seed produced by these transgenic plants, methods for producing the transgenic plants, and methods for increasing tolerance of a plant to water deprivation. The transgenic plants express plant transcription factor polypeptides first identified in *Arabidopsis thaliana*, a plant that is widely used as a model species.
3. For the purposes of this declaration, a plant "line" means the progeny (through seed or vegetative propagation) of a transformation event or a newly bred variety (specific genotype).

4. The present application provides methods for analysis and identification of sequences from diverse species that are closely related. These methods include phylogenetic analysis, sequence alignments and percentage identity determination.

Exhibit A, filed February 13, 2006 with a response to a previous Office action, provided a number of sequences that are closely related to G1274 and fall within, or just outside of, the G1274 clade of transcription factor polypeptides. These sequences derive from diverse species that include dicots *Arabidopsis thaliana* and *Glycine max* and monocots *Oryza sativa* and *Zea mays*. The G1274 clade polypeptides comprise conserved domains, indicated by the underlined residues in Exhibit A, that are at least 74% identical to the conserved domain of G1274, amino acid coordinates 111-164.

Sequences that lie just outside of the clade also comprise conserved domains similar to that found in G1274, but these are less similar to G1274 than the sequences within the clade. For example, G2517, G194, and G1758, have conserved domains that are 55% to 61% identical to the conserved domain of G1274.

5. This declaration summarizes and updates some of the data obtained with sequences presented in the previous declaration and in Exhibit A, submitted February 13, 2006. Based on experimental analysis, plants overexpressing sequences that have conserved domains 57% or more identical to the conserved domain of amino acid coordinates 111-164 of G1274 were found to be more tolerant to water deprivation-based treatments than control plants. This trait was observed for all of the G1274-related sequences transformed into plants that fall within the scope of the claims and that were comprehensively tested in water deprivation assays. These results are summarized in Table 1. The data presented in Table 1 include positive assay results from plate-based severe dehydration assays or soil-based drought assays. Transgenic plant lines that showed significant evidence of tolerance to severe desiccation, drought, or, as noted, a related assay (e.g., hyperosmotic sucrose treatment) were considered to be positive indicators affirming the ability of these sequences to confer water deprivation tolerance in the plants. Not included in Table 1 are data for several sequences introduced into transgenic plants for which water-deprivation results are presently considered inconclusive, as only a few lines were generated and/or not all water deprivation-related assays, including soil drought assays, have been performed.

6. Severe desiccation and soil drought assays were performed according to the method described in Exhibit C, dated February 13, 2006.

Sucrose assays were by surface sterilizing seeds as provided in previously submitted Exhibit C. The seeds were then sown on conditional media with a basal composition of 80% MS + Vitamins plus 9.4% sucrose. The plates were incubated at 22 °C under 24-hour light ($120\text{-}130\ \mu\text{E m}^{-2}\text{ s}^{-1}$) in a growth chamber. Evaluation of germination and seedling vigor was performed five days after planting.

Table 1. Water deprivation assay data summary for plants overexpressing G1274 and closely related sequences

Transcription factor (TF) Gene Identifier	Sequence identifier in MBI-0054 Table 7 (SEQ ID NO: or accession no.)	Species from which TF is derived	%ID of TF conserved domain to conserved domain of G1274	Assay Results	Performance of specific promoter-TF combinations in transgenic <i>Arabidopsis</i> plants
G1274	194	<i>Arabidopsis thaliana</i>	100%	+	<ul style="list-style-type: none"> • Seven 35S:: G1274 <i>Arabidopsis</i> lines (7 of 7 lines tested against wild-type controls) were more tolerant and recovered better from drought than controls in soil-based drought assays • Four 35S:: G1274 <i>Arabidopsis</i> lines (4 of 29 lines tested) were more tolerant to severe desiccation in plate-based assays than controls • Two 35S:: G1274 <i>Arabidopsis</i> lines (2 of 29 lines tested) were more tolerant to sucrose in plate-based assays than controls • Three STM::G1274 lines (shoot apical meristem-specific promoter; 3 of 10 lines tested) were more tolerant to severe desiccation in plate-based assays than controls • One STM::G1274 line (shoot apical meristem-specific promoter; 1 of 3 lines tested) recovered better from a soil-based drought treatment than controls • Four ARSK1::G1274 lines (root-specific promoter; 4 of 10 lines tested) were more tolerant to severe desiccation in plate-based assays than controls • Three SUC2::G1274 lines (vascular-specific promoter; 3 of 10 lines tested) were more tolerant to severe desiccation in plate-based assays than controls • One RD29a::G1274 line (stress-inducible promoter; 1 of 3 lines tested) recovered better from drought than controls

G3724	969	<i>Glycine max</i>	83%	+	<ul style="list-style-type: none"> • Two 35S::G3724 <i>Arabidopsis</i> lines (2 of 3 lines tested) were more tolerant to drought than controls in soil-based drought assays; one of these lines also recovered better from drought than controls • Two 35S::G3724 lines (2 of 10 lines tested) were more tolerant to severe desiccation in plate-based assays than controls • Three 35S::G3724 lines (3 of 10 lines tested) were more tolerant to sucrose than controls
G3804	974	<i>Zea mays</i>	81%	+	<ul style="list-style-type: none"> • Three 35S::G3804 <i>Arabidopsis</i> lines (3 of 3 lines tested) were more tolerant to drought, and recovered better from drought, than controls *
G3803		<i>Glycine max</i>	79%	+	<ul style="list-style-type: none"> • Four 35S::G3803 <i>Arabidopsis</i> lines were more tolerant to severe desiccation in plate-based assays (4 of 10 lines tested; each line was positive once in two assays performed in a total of 20 assays)
G3721		<i>Oryza sativa</i>	77%	+	<ul style="list-style-type: none"> • Three 35S::G3721 <i>Arabidopsis</i> lines (3 of 3 lines tested) were more tolerant to drought, and recovered better from drought, than controls
G3722	975	<i>Zea mays</i>	77%	+	<ul style="list-style-type: none"> • Of 10 lines tested, one 35S::G3722 line was more tolerant to both severe desiccation and sucrose, a second line was more tolerant to sucrose, and a third line was more tolerant to severe desiccation in plate-based assays
G3726	971	<i>Oryza sativa</i>	77%	+	<ul style="list-style-type: none"> • Two 35S::G3726 <i>Arabidopsis</i> lines (2 of 3 lines tested) were more tolerant to drought, and recovered better from drought, than G912-overexpressing controls *


G1275		<i>Arabidopsis thaliana</i>	75%	+	<ul style="list-style-type: none"> • Two 35S::G1275 lines (2 of 8 lines tested) were more tolerant than controls to sucrose in plate-based assays • Two CUT1::G1275 lines (epidermal-specific promoter; 2 of 3 lines tested) recovered better from drought than controls • Three CUT1::G1275 lines (epidermal-specific promoter; 3 of 10 lines tested) were more tolerant to sucrose than controls in plate-based assays • Two AS1::G1275 lines (emergent leaf primordia-specific promoter; 2 of 10 lines tested) were more tolerant to severe desiccation than controls in plate-based assays • Two STM::G1275 lines (shoot apical meristem-specific promoter; 2 of 10 lines tested) were more tolerant to severe desiccation in plate-based assays than controls • Two SUC2::G1275 lines (vascular-specific promoter; 2 of 10 lines tested) were more tolerant than controls to sucrose in plate-based assays • Two RD29A::G1275 <i>Arabidopsis</i> lines (stress-inducible promoter; 2 of 3 lines tested) were more tolerant to drought than controls in soil-based drought assays; one of these lines also recovered better from drought than controls • Two RD29A::G1275 <i>Arabidopsis</i> lines (stress-inducible promoter; 2 of 10 lines tested) were more tolerant to severe desiccation in than controls plate-based assays
G3729		<i>Oryza sativa</i>	74%	+	<ul style="list-style-type: none"> • One 35S::G3729 line (1 of 10 tested) was more tolerant than controls to both sucrose and severe desiccation in plate-based assays

G2517		<i>Arabidopsis thaliana</i>	61%	+	<ul style="list-style-type: none"> • Six 35S::G2517 lines (6 of 10 tested) were more tolerant in severe desiccation assays than controls
G194		<i>Arabidopsis thaliana</i>	57%	+	<ul style="list-style-type: none"> • Four 35S::G194 lines (4 of 4 tested) were more tolerant in severe desiccation assays than controls
G1758		<i>Arabidopsis thaliana</i>	55%	Wild-type performance or -	<ul style="list-style-type: none"> • Two 35S::G1758 lines were no more tolerant to drought and one line was less tolerant to drought than controls in soil-based assays (3 lines examined)

- + indicates greater tolerance to drought in soil-based assays, or severe desiccation or sucrose in plate-based assays, as compared to performance of wild-type or empty vector controls (except as noted*);
data for desiccation and sucrose assays based on visual comparison to control seedlings
data for drought assays significant at $p < 0.11$
- indicates less tolerant than controls in a water deprivation-related assay
- * results of particular and noteworthy significance; control plants used in these experiments overexpressed G912, a CBF transcription factor, and thus the controls were more drought-tolerant in soil-based assays than wild-type

7. I hereby declare that all statements made herein are true and that they are based on my own knowledge, information and belief. These statements are made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issued from it.

Date: June 26, 2006


Peter Repetti, Ph.D.
Senior Scientist
Mendel Biotechnology, Inc.

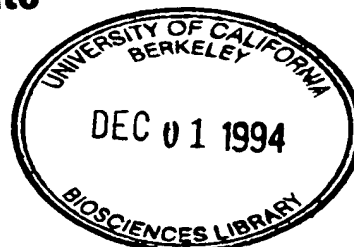
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ORIGINAL PAPER

Sumie Ishiguro · Kenzo Nakamura

Characterization of a cDNA encoding a novel DNA-binding protein, SPF1, that recognizes SP8 sequences in the 5' upstream regions of genes coding for sporamin and β -amylase from sweet potato

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Abstract We isolated a cDNA encoding a DNA-binding protein, SPF1, of sweet potato that binds to the SP8a (ACTGTGTA) and SP8b (TACTATT) sequences present in the 5' upstream regions of three different genes coding for sporamin and β -amylase of tuberous roots. SPF1 comprises 549 amino acids and is enriched in both basic and acidic residues. The amino acid sequence of SPF1 shows no significant homology to any known protein sequences, suggesting that it may represent a new class of DNA-binding protein. Binding studies with ^{35}S -labeled SPF1, synthesized *in vitro*, and synthetic DNA fragments indicated that, although SPF1 binds to both the SP8a and SP8b sequences, it binds much more strongly to SP8a than to SP8b. SPF1 bound to the SP8a sequence as a monomer. The DNA-binding domain of SPF1 was localized within the C-terminal half of this protein, and a 162-amino acid fragment of SPF1 (Met³¹⁰–Arg⁴⁷²) showed DNA-binding activity with no change in target sequence specificity. This fragment contains a region enriched in basic amino acids adjacent to a highly acidic stretch. A sequence which is highly homologous to a 40-amino acid sequence in the basic region of the DNA-binding domain is duplicated in the N-terminal part of SPF1. The gene coding for SPF1 is present in one or a few copies per haploid genome and the SPF1 mRNA was detected in leaves, stems and tuberous roots of the sweet potato, in addition to petioles. The level of SPF1

mRNA in the petioles decreased when leaf-petiole cuttings were treated with sucrose to induce accumulation of sporamin and β -amylase mRNAs.

Key words Transcription factor · Coordinated regulation · DNA binding domain · Sweet potato

Introduction

Transcriptional regulation of gene expression is directed by the action of specific transcription factors which interact with regulatory elements residing in the promoter region of the corresponding gene. In groups of genes that are induced coordinately in response to external and internal stimuli, the binding of key transcription factors to conserved *cis*-regulatory elements in promoters of these genes seems to be essential for coordination of the activation of their transcription. However, in plants, only a few factors have been shown to carry out such a role.

Sporamin and β -amylase are two major proteins in tuberous roots of the sweet potato (*Ipomoea batatas* L.) which are absent, or present only in very small amounts, in organs other than the tuberous root. In addition to this developmental regulation, the members of the sporamin multigene family (Hattori and Nakamura 1988; Hattori et al. 1989) and the β -amylase gene (Yoshida et al. 1992) show concomitantly regulated ectopic expression in organs other than the tuberous roots under certain conditions. They are highly expressed in stems when sweet potato plantlets are cultured axenically on sucrose medium (Hattori et al. 1990; our unpublished results). Concomitant induction of expression of the members of sporamin multigene family and the β -amylase gene occurs in leaves and petioles following exogenous supply of high concentrations of sucrose and other metabolizable sugars (Hattori et al. 1991; Nakamura et al. 1991), or of low concentrations of oligosaccharides such as polygalacturonic acid (PGA) and chitosan (Ohto et al. 1992). It

The nucleotide sequence reported in this paper has been submitted to the EMBL/GenBank/DBJ database under the accession number D30038

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has been suggested that coordinated regulation of the members of the sporamin multigene family and the β -amylase gene (β -Amy) is mediated by one or more common transcription factor(s).

Studies *in vitro* on the binding of nuclear extracts, prepared from the sweet potato, to the 5' upstream regions of the two genomic clones for sporamin characterized so far (gSPO-A1 and gSPO-B1; Hattori et al. 1988) and of the β -Amy gene (Yoshida et al. 1992) identified multiple activities in the extract, which bind to different regions of the 5' upstream regions of these genes (Ishiguro and Nakamura 1992; Ishiguro et al. 1993). Among these activities, we have previously characterized an activity (SP8BF) that showed binding to the 5'-upstream regions of all three genes examined (Ishiguro and Nakamura 1992). SP8BF binds to two conserved sequences, the SP8a sequence (ACTGTGTA) and the SP8b sequence (TACTATT) in the 5' upstream regions of these genes. Within the 5' upstream regions examined in these studies, gSPO-A1 and gSPO-B1 contained one SP8a site and three SP8b sites, respectively. On the other hand, β -Amy contained one SP8a site and two SP8b sites. It was suggested that SP8BF may act as a key factor regulating coordinated expression of the members of sporamin multigene family and the β -amylase gene. Sequences similar to the SP8 motifs are present in the regulatory region of several other plant genes (Ishiguro and Nakamura 1992).

In this paper, we characterize a sweet potato cDNA encoding a protein, SPF1, that binds to both of the SP8a and SP8b sequences, although with significantly different affinities. The amino acid sequence of SPF1 suggests that it represents a novel DNA-binding protein.

Materials and methods

Plant materials

Sweet potatoes (*Ipomoea batatas* Lam. cv. Kokei No. 14) were grown at the Nagoya University Experimental Farm. Treatment of leaf-petiole cutting of sweet potato with either sucrose or polygalacturonic acid (PGA) were carried out as described previously (Nakamura et al. 1991; Ohto et al. 1992).

Standard molecular biology techniques

Basic molecular biology techniques were carried out according to Sambrook et al. (1989). Methods for the isolation of total DNA from leaves of sweet potato and Southern blot analysis (Yoshida et al. 1992) and those for the isolation of total RNA from the plant tissues for Northern blot hybridization (Nakamura et al. 1991) were performed as described previously. DNA fragments were labeled by random priming method using a commercial kit (Amersham) and [γ - 32 P]dCTP, and used as hybridization probes. The structural analysis and database search of the cDNA were done using Genex-CD (Software Development, Tokyo).

Oligonucleotides

All oligonucleotides were synthesized on a DNA synthesizer (Model 381A, Applied Biosystems). Oligonucleotides used in the screening of cDNA expression library and in the DNA-binding studies were synthesized with complementary overhangs (see Fig. 4A) and were ligated unidirectionally to form multimers of 3–6 units in suitable plasmids. Multimers of the oligonucleotides were excised from the vector with restriction endonucleases and purified by gel electrophoresis.

Construction and screening of cDNA expression library

Total RNA was isolated from the petiole portions of leaf-petiole cuttings that had been treated with 6% sucrose for 60 h under continuous illumination. Poly(A)⁺ RNA was prepared on Oligotex-dT 30 (Takara Shuzo, Kyoto) from the total RNA. From 2 μ g of poly(A)⁺ RNA, oligo(dT)-primed cDNAs were synthesized using a commercially available cDNA synthesis kit (Promega) and a λ gt11 library was constructed using an *in vitro* packaging extract (Gigapack Gold, Stratagene).

Screening of the λ gt11 library was performed according to Singh et al. (1989) with the following modifications. Nitrocellulose replica filters (BA 85, Schleicher and Schuell) of plates containing about 30 000 plaques were prepared in duplicate to distinguish true positives from noise. The filters were treated for 60 min with the blocking solution (50 mM HEPES-KOH pH 7.9, 25 mM KCl, 0.5 mM EDTA, 1 mM DTT, 5% non-fat dry milk). After the filters were rinsed with the binding buffer (25 mM HEPES-KOH pH 7.9, 25 mM KCl, 0.5 mM EDTA, 1 mM DTT), the binding reaction was performed for 1 h at room temperature in the binding buffer supplemented with 5 μ g/ml of the denatured, sonicated salmon testis DNA and 6×10^5 cpm/ml of the radiolabeled DNA probe. A fragment comprising six copies of the oligo-SP8a sequence (Fig. 4A; Ishiguro and Nakamura 1992) was labeled with [α - 32 P]dCTP by nick translation and used as a probe. The filters were washed for 20 min in binding buffer before autoradiography.

Construction of full-length cDNA for SPF1

From 5 μ g of poly(A)⁺ RNA, first-strand cDNA was synthesized by priming with the SPF1-specific primer, CTAGTGGAC-TGAGGCTTC, corresponding to nucleotides 843 to 826 in the antisense strand of full-length SPF1 (see Fig. 2.) The cDNA library was constructed in the λ ZAPII vector (Stratagene) and screened by hybridization with an 0.56 kb 32 P-labeled *Eco*RI fragment of clone D1 (see Fig. 1). One of the clones recovered, λ K15, contained cDNA covering the N-terminal part of SPF1. A plasmid carrying the whole cDNA insert of λ K15 was obtained by an *in vivo* excision procedure. The resulting plasmid DNA was cleaved with *Pst*I and *Bam*HI. The 1.1 kb *Pst*I-*Xho*I fragment bearing the coding sequence and the 0.45 kb *Xho*I-*Bam*HI fragment covering the 3'-noncoding region were isolated from the clone D1. Ligation of three DNA fragments generated a full-length cDNA of SPF1, pSPF1. The whole insert of pSPF1 was re-excised from the vector and inserted into sites located downstream of the SP6 promoter in the pSP64 vector to yield pSP-SPF1 (Fig. 1).

Synthesis of SPF1 polypeptide *in vitro*

The pSP-SPF1 plasmid DNA was linearized with appropriate restriction endonucleases, and transcribed with SP6 RNA polymerase, using a MEGAscript *in vitro* transcription kit (Ambion) in the presence of diguanosine triphosphate. For the generation of

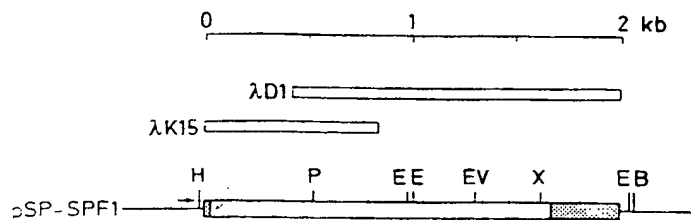


Fig. 1 Structures of cDNA clones for SPF1. Structures of two partial cDNA clones λ D1 and λ K15 and the full-length cDNA for SPF1 reconstituted from these two partial clones in pSP64 vector are shown. The following abbreviations are used for the restriction enzymes: H, *Hind*III; P, *Pst*I; E, *Eco*RI; EV, *Eco*RV; X, *Xho*I; B, *Bam*HI. In the structure of pSP-SPF1, the location of SP6 promoter is indicated by an arrow, and the 5'- and 3'-untranslated regions are shaded.

N-terminally truncated SPF1, we synthesized two 5' primers carrying, in series, an SP6 promoter sequence and either AG-CCTTCCCCCTGTGACTACT, or AGTCGTACTGGTCTAT-TGGTAAC, which correspond to the sequences immediately upstream of the codons for Met¹⁸⁰ or Met³¹⁰, respectively. An oligonucleotide, TGCCAATTACCCAGACAC, corresponding to a sequence within the 3'-untranslated region of SPF1 cDNA was used as a 3' primer. Using these primers, a total of 25 PCR cycles was performed. The PCR products were used directly for the *in vitro* transcription. The *in vitro* transcripts were translated using wheat germ extract in 10 μ l reactions with 1.1 MBq (47TBq/mM) [³⁵S]methionine according to Erickson and Blobel (1983).

DNA-binding assay

The DNA-binding assay utilizing SPF1 synthesized *in vitro* is based on the procedures described by Hope and Struhl (1985). Binding was carried out at 25°C for 30 min in 12 μ l of a reaction mixture composed of 25 mM HEPES-KOH pH 7.9, 1 mM DTT, 50 mM KCl, 0.5 mM EDTA, 7.5% glycerol, 3 μ g poly(dI-dC)·poly(dI-dC), appropriate amounts of oligonucleotide multimers described in the Results, and the translation mixture. SPF1 synthesized *in vitro* was used without further purification. The reaction mixtures were loaded onto 5% polyacrylamide gel containing 45 mM Tris-borate buffer (pH 8.3) and 1 mM EDTA. After the electrophoresis at 4°C, signals were detected by fluorography of the gel and band intensities were subjected to densitometry.

Results

Molecular cloning of a factor that binds to the SP8a sequence

In order to isolate a cDNA for Sp8BF, we constructed a λ gt11 cDNA expression library from poly(A)⁺ RNAs prepared from sweet potato petioles that had been treated with 6% sucrose for 60 h to induce expression of the genes encoding sporamin and β -amylase. The library, which contained about 1×10^5 independent clones, was screened for binding of expressed proteins to multimers of the oligo-SP8a probe (see Fig. 4A; Shiguro and Nakamura, 1991) and one positive clone was isolated. This plaque did not show binding to the unrelated oligonucleotide oligo-3231 (see Fig. 4A). The

cDNA insert of this clone, designated D1, was about 1.6 kb in length (Fig. 1). Nucleotide sequencing of the 5'-terminal part of D1 indicated that it has an open reading frame that is in frame to the *lacZ* gene of the vector. In the Northern blot analysis of the petiole RNAs, an RNA hybridized with the ³²P-labeled D1 probe, but was longer than the D1 cDNA (data not shown). These results indicated that the D1 cDNA is missing the 5'-portion of the corresponding mRNA sequence.

In order to isolate a cDNA that includes the 5' portion of the mRNA missing in the D1 cDNA, we constructed another cDNA library using the sequence of the 5' region of the D1 cDNA as primer (see Fig. 2) for the synthesis of cDNA. Using the D1 cDNA as a probe, a clone, K15, was isolated from this library. The K15 cDNA contained a 422-bp sequence that overlaps with the 5' part of the D1 cDNA and a further 417-bp sequence 5' to it (Figs. 1, 2). Restriction enzyme fragments from the K15 and D1 cDNAs were combined to construct a full-length cDNA and the protein encoded by this cDNA was designated SPF1 (Fig. 1).

The SPF1 cDNA is 1993 bp in length and it can code for a polypeptide with 549 amino acids (Fig. 2). The SPF1 is a highly hydrophilic protein enriched in both basic and acidic residues (Fig. 3A, B). The amino acid sequence of SPF1 contained a duplication of a 40-amino acid sequence: the sequence of residues 210–249 is 75% identical to that of residues 386–425 (Fig. 3C). We could not find any DNA or protein sequence that shared significant homology with the SPF1 cDNA or protein in the sequence databases from EMBL, GenBank, and DDBJ, indicating that SPF1 represents a novel class of sequence-specific DNA-binding protein.

SPF1 binds to SP8a and SP8b sequences with significantly different affinities

In order to examine further the target site sequence specificity of SPF1, we carried out a gel mobility shift assay, using SPF1 proteins prepared by *in vitro* translation of SP6 transcripts of pSP-SPF1 cDNA in a wheat germ extract, and ³²P-labeled DNA probes. However, we could not detect formation of a complex of SPF1 protein and the DNA probes. We assume that the level of SPF1 protein synthesized *in vitro* was not sufficient for formation of detectable amounts of complex in this assay. Alternatively, it is possible that the signal of SPF1-DNA complex was masked by the strong signal due to endogenous SP8BF activity, which is present in wheat germ extracts (data not shown).

We then carried out DNA-binding assays with ³⁵S-labeled SPF1 synthesized *in vitro* in a wheat germ extract. The ³⁵S-labeled SPF1 protein did not enter a 5% polyacrylamide gel on electrophoresis using Tris-borate buffer (pH 8.3) and 1 mM EDTA (Fig. 4B,

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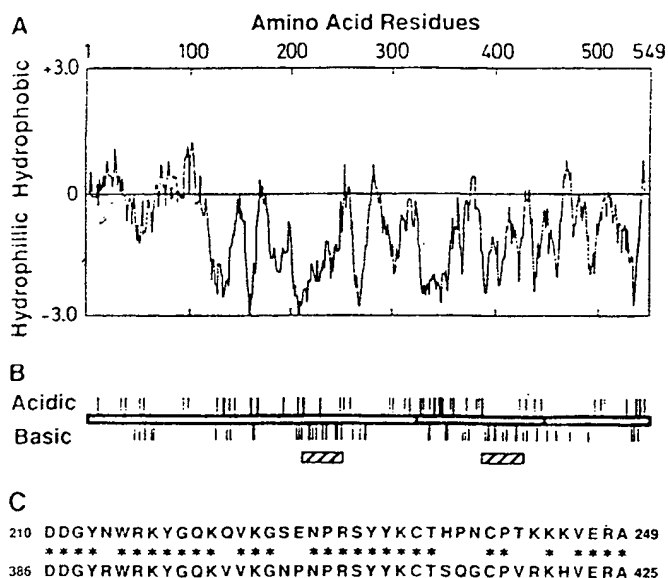


Fig. 3A–C Structural features of SPF1. **A** Hydropathy analysis performed according to Kyte and Doolittle (1982) using a window of nine amino acids. **B** Distribution of acidic (Asp and Glu) and basic (Lys and Arg) amino acid residues in SPF1 polypeptide. The 40-amino acid repeated sequence is indicated by hatched boxes. The location of the M310-R472 polypeptide that showed DNA-binding activity is shaded. **C** The 40 amino acids-long basic repeat sequence in SPF1. Numbers are as in Fig. 2. Identical amino acid residues are indicated by asterisks

lane 1). The ^{35}S -labeled SPF1 protein was mixed with the increasing amounts of oligo-SP8a multimers, and subjected to gel electrophoresis. Unlike free SPF1, several bands of ^{35}S -labeled SPF1 protein appeared in the gel upon electrophoresis (Fig. 4B, lanes 2 to 4). The intensity of the most prominent band of radioactivity (arrowhead in Fig. 4B) increased with the amount of DNA added to the reaction mixture (Fig. 4C). When the ^{35}S -labeled SPF1 protein was mixed with multimers of oligo-3231, only a faint band of radioactivity

appeared in the gel (Fig. 4B, lanes 14 to 16, and Fig. 4C). These results indicate that the major band of radioactivity in the gel is due to sequence-specific binding of SPF1 and represents ^{35}S -labeled SPF1 complexed with DNA. Several minor bands also appeared in the gel, and the nature of these minor bands is not known at present.

SPF1 also bound to the oligonucleotide oligo-SP8b multimer containing the SP8b sequence (Fig. 4B, lanes 8 to 10). However, the binding of SPF1 to oligo-SP8b was significantly weaker than that with oligo-SP8a and approximately 25 times more DNA was required to

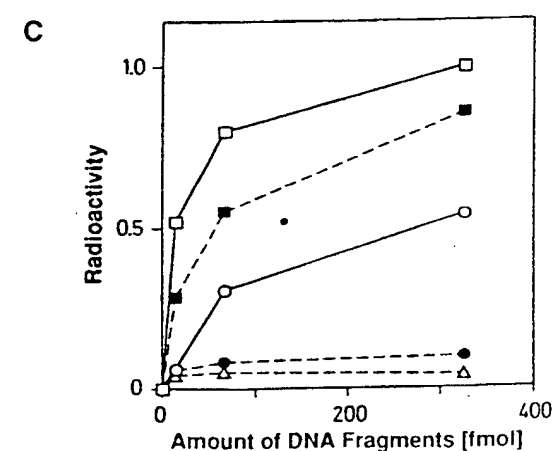
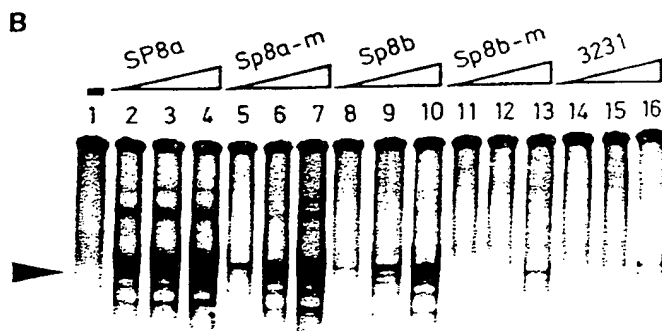
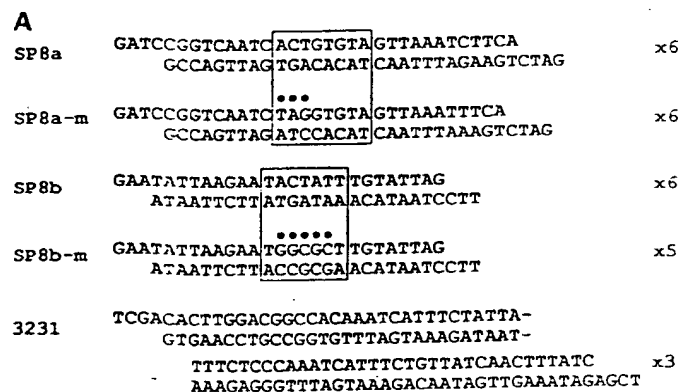


Fig. 4A–C Binding of SPF1 synthesized *in vitro* to various DNA fragments. **A** Nucleotide sequences of oligonucleotides used. Multimers comprising six copies of oligo-SP8a, oligo-SP8a-m, oligo-SP8b five copies of oligo-SP8b-m and three copies of oligo-3231 respectively were used for gel retardation studies. The SP8a sequence in the oligo-SP8a and the SP8b sequence in the oligo-SP8b are boxed. Nucleotides in the oligo-SP8a-m and oligo-SP8b-m that are different from the oligo-SP8a and oligo-SP8b, respectively, are indicated by dots. **B** Fluorograph of a gel retardation analysis. The ^{35}S -labeled SPF1 protein obtained by translation *in vitro* was mixed with increasing amounts of the oligonucleotide multimers indicated. After incubation at 25°C for 30 min, the reaction mixtures were subjected to electrophoresis through a polyacrylamide gel. The arrowhead indicates the position of the major SPF1-DNA complex. **C** The amount of ^{35}S radioactivity in the major band indicated by the arrowhead in B was estimated by densitometry of the fluorograph. open squares, oligo-SP8a; filled squares, oligo-SP8a-m; open circles, oligo-SP8b; filled circles, oligo-SP8b-m; open triangles, oligo-3231

produce SPF1-DNA complex with the same band intensity (Fig. 4B, lanes 2 and 10, and Fig. 4C). The binding of SPF1 with oligo-SP8a-m, which contains mutations in the SP8a sequence (Fig. 4A) was about five times weaker than the binding with oligo-SP8a (Fig. 4B, lanes 5 to 7, and Fig. 4C). The weak binding of the oligo-SP8a-m could be due to the presence of an ACTA core sequence of the SP8 consensus sequence (Ishiguro and Nakamura 1992) in the opposite strand from the SP8a sequence. Mutations in the SP8b sequence in oligo-SP8b-m (Fig. 4A) significantly reduced the binding with SPF1 (Fig. 4B, lanes 11 to 13, and Fig. 4C).

The DNA-binding domain is localized in the C-terminal half of SPF1

To identify the DNA-binding domain in the sequence of SPF1, we generated a series of truncated SPF1 polypeptides *in vitro* and assayed for their ability to bind with the oligo-SP8a multimer. We first prepared C-terminally deleted derivatives of SPF1 by restriction enzyme cleavage of the SPF1 transcription template at various positions within the coding region of SPF1 (Fig. 5A). Cleavage of SPF1 cDNA with *Bam*HI allows synthesis of full-length, SPF1 (referred to below as M1-A549 for the N-terminal Met¹ and the C-terminal Ala⁵⁴⁹ residues). Cleavage of cDNA with *Nhe*I results in truncation of the C-terminal amino acid of SPF1 to produce M1-L548 (Fig. 5A) and did not affect the binding activity of SPF1 (Fig. 5B, lanes 1 and 2).

The M1-R448 protein generated by cleavage of cDNA with *Ava*I still showed the binding with oligo-SP8a, although the activity was significantly lower than with M1-A549 or M1-L548 (Fig. 5B, lane 3). The mutant SPF1 protein M1-H421, generated by truncation at the *Pma*CI site of the cDNA, did not bind to the DNA fragment (Fig. 5B, lane 4). Truncations at the *Eco*RI (M1-N318) or *Pst*I (M1-S167) sites of the cDNA also produced mutant polypeptides that had lost the DNA-binding activity (data not shown).

To generate SPF1 proteins with N-terminal truncations, we used ATG codons for internal methionine residues. Met¹⁸⁰ and Met³¹⁰, as translational start codons. In order to delete the nucleotide sequence of the 5'-terminal part of SPF1 cDNA, we used PCR amplification mutagenesis. As a 5' primer, we synthesized oligonucleotides comprising the 36 nucleotide SP6 promoter sequence at the 5'-terminus followed by the sequence immediately upstream of the ATG codon for Met¹⁸⁰ or Met³¹⁰. An oligonucleotide that is complementary to the sequence in the 3'-noncoding region of SPF1 cDNA was used as a 3' primer. By these methods we generated two N-terminally truncated SPF1 proteins, M180-A549 and M310-A549 (Fig. 5A). Although

translation from the ATG codon of Met³¹⁰ was as efficient as translation from the initiator codon of the wild-type SPF1, translation from the ATG codon of Met¹⁸⁰ was less efficient (data not shown). Both of the N-terminally truncated proteins M180-A549 and M310-A549 showed binding with the oligo-SP8a DNA multimer (Fig. 5B, lanes 5 and 6). It is important to mention that the bands in lanes 5 and 6 are comparable in intensity despite the lower translational efficiency of the mRNA for M180-A549. These results suggest that the DNA-binding activity of M180-A549 is higher than that of M310-A549.

We also prepared SPF1 polypeptides with both N- and C-terminal truncations by cleavage of the N-terminally truncated cDNA with restriction enzymes. By cleaving the M310 DNA with *Sma*I, we obtained M310-R472, which showed binding to the SP8a sequence (Fig. 5B, lane 7). However, further truncation of the C-terminus of M310-R472 to M310-R448 by cleavage of the M310 DNA with *Ava*I resulted in loss of the DNA-binding activity. The sequence specificity of the binding of the truncated polypeptide M310-R472, as examined with oligo-SP8a, oligo-SP8b, and oligo 3231 multimers, did not change from that of the full-length protein (data not shown). From these results, we conclude that the DNA-binding domain of SPF1 is located within the sequence from Met³¹⁰ to Arg⁴⁷² of this protein.

SPF1 binds to DNA as a monomer

The electrophoretic mobilities of protein-DNA complexes formed by SPF1 and M310-A549 differ significantly (Fig. 5B, lanes 1 and 6). We co-translated SP6 RNAs for SPF1 and for the M310-A549 in the same reaction, and analyzed the products in the DNA-binding assay with oligo-SP8a sequence. Two major bands of protein-DNA complexes, corresponding to the protein-DNA complexes formed with SPF1 or M310-A549, appeared on the gel (Fig. 5B, lane 1 + 6). No additional protein-DNA complexes, which would be indicative of the formation of multimers, were observed, suggesting that SPF1 binds to the SP8 sequences as a monomer.

Southern and Northern blot analyses of SPF1

Genomic Southern blots of sweet potato DNA that had been digested with several restriction endonucleases were hybridized with a cDNA fragment corresponding to nucleotide positions 418-979 of SPF1. Only one or a few bands hybridized with the probe in each restriction digest (data not shown), indicating that SPF1 is encoded by a single-copy gene or a small gene family in the haploid genome of the sweet potato.

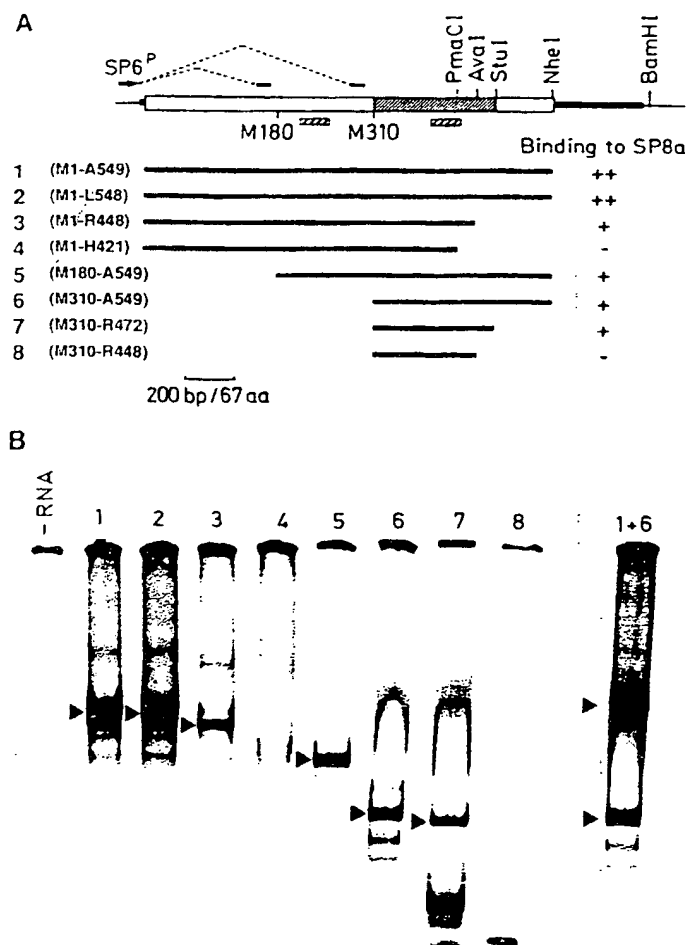


Fig. 5A, B Localization of the DNA-binding domain of SPF1. A Structures of truncated SPF1 polypeptides. The structure of the SPF1 cDNA is shown schematically at the top. The coding region is indicated by an open bar with the shaded bar indicating the location of M310-R472 polypeptide that showed DNA-binding activity. The 40-amino acid repeated sequence is indicated by hatched boxes. Locations of restriction enzyme cleavage sites used to generate the C-terminal truncations and Met¹⁸⁰ and Met³¹⁰ residues used to generate the N-terminal truncations are indicated. B Fluorograph of a gel retardation analysis. Number of lanes correspond to the truncated versions of SPF1 shown in A. The truncated, ³⁵S-labeled polypeptides were mixed with oligo-SP8a multimers and subjected to electrophoresis through a polyacrylamide gel. The major protein-DNA complexes are indicated by arrowheads.

Northern blot analysis demonstrated that SPF1 mRNA is present in all of the organ systems of the sweet potato examined: leaves, petioles, stems and tuberous roots (Fig. 6A). The level of SPF1 mRNA seemed to be higher in leaves and tuberous roots than in stems and petioles. Figure 6B shows that the level of SPF1 mRNA in the petioles decreased after treatment with sucrose or PGA. Hybridization of the same blot with the β -amylase cDNA probe detected a β -amylase mRNA in samples of RNA from petioles that had been treated with sucrose or PGA, but not in the RNA from non-treated petioles (data not shown).

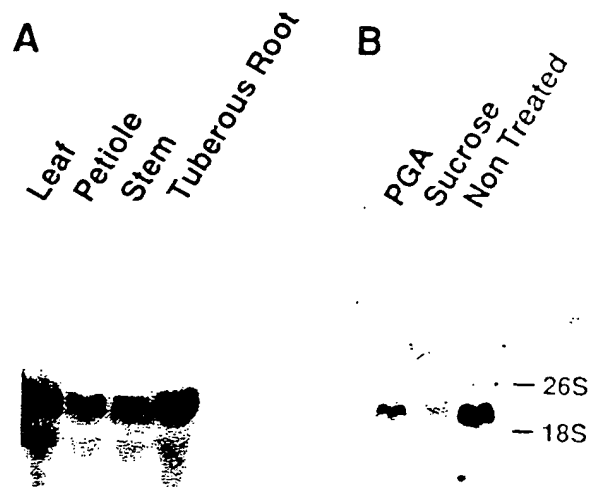


Fig. 6A, B Northern blot hybridization of RNA isolated from various tissues of sweet potato. A Total RNAs (25 μ g in each lane) isolated from leaf, petiole, stem and tuberous root of the field-grown sweet potato were analyzed. B Total RNAs (20 μ g in each lane) isolated from petioles that had been treated with 0.1% PGA or 6% sucrose for 12 h in the dark were analyzed, together with total RNA isolated from non-treated petioles.

Discussion

In this study, we isolated and characterized a sweet potato cDNA encoding a protein, SPF1, that binds to the SP8 sequence motif conserved in the 5'-upstream regions of three genes encoding sporamin and β -amylase of tuberous roots (Ishiguro and Nakamura 1992). Sequences similar to SP8 motifs are also present in the regulatory regions of several other plant genes (Ishiguro and Nakamura 1992). Based on the absence of significant sequence similarity to any protein currently in the databases, SPF1 represents a novel DNA-binding protein.

SPF1 is composed of 549 amino acids and enriched in both basic and acidic amino acid residues (Figs. 2 and 3). The DNA-binding domain of SPF1 is localized within the C-terminal half of the protein and the 163-amino acid fragment Met³¹⁰ to Arg⁴⁷² retained DNA-binding activity, although the activity was less than that of the whole protein (Fig. 5). Truncation of SPF1 after His⁴²¹ (M1-H421) resulted in the loss of DNA-binding activity. The C-terminal truncation M1-R448 still retained the DNA-binding activity, however

by contrast, the C-terminal truncation of the M310 derivative of SPF1 after Arg⁴⁴⁸ (M310-R448) eliminated the activity. This difference suggests that the N-terminal structure may affect the folding of the DNA-binding domain of SPF1. The sequence of M310-R472 contains a highly acidic stretch and a basic region in the N-terminal and C-terminal parts, respectively (Fig. 3). In many transcription factors, the DNA-binding domains are localized in the basic region. The basic region within the M310-R472 polypeptide may play an important role in the binding of SPF1 to DNA.

Most sequence-specific DNA-binding proteins bind to their cognate DNA via structural domains that make sequence-specific contacts with the DNA bases in the major groove. Many conformational structures, such as leucine-zippers, Cys-Cys or Cys-His zinc-fingers, helix-turn-helix and helix-loop-helix motifs have been identified in the DNA-binding domains of proteins, which are responsible for the recognition of specific DNA sequences (for reviews see Churchill and Travers 1991; Harrison 1991; Struhl 1989). The DNA-binding domain of SPF1 has neither leucine residues arranged to form an amphiphilic α -helix nor cysteine residues that might be arranged as Cys-Cys or Cys-His zinc fingers.

A sequence highly homologous to a 40 amino-acid sequence in the basic region within the DNA-binding domain of SPF1, (Asp³⁸⁶-Ala⁴²⁵), is also present in the N-terminal part of SPF1 (Asp²¹⁰-Ala²⁴⁹; Fig. 3). These two sequences share amino acid sequence identity of 75%. The C-terminal truncation after His⁴²¹ that is located within the C-terminal basic repeat eliminates the DNA-binding activity of SPF1 (Fig. 5B, lane 4), suggesting that the C-terminal basic repeat sequence constitutes part of the DNA-binding domain of SPF1. By contrast, the C-terminally truncated polypeptides of SPF1 that have only the N-terminal basic repeat, such as M1-H421 (Fig. 5B, lane 4) or M1-N318 (data not shown), do not show binding to oligo-SP8a. Furthermore, the DNA-binding activity of SPF1 is retained after elimination of the N-terminal basic repeat (M310-A549 and M310-R472, Fig. 5B, lanes 6 to 7). These results suggest that the N-terminal basic repeat does not play an essential role in the DNA-binding activity of SPF1. However, we can not exclude the possibility that the N-terminal basic repeat may also mediate DNA-binding activity with a target site sequence specificity different from that of the C-terminal basic repeat. Recently, the transcription factor GT-2 of rice, which binds to GT motifs in the phytochrome gene, has been shown to bear two independently functioning DNA-binding domains with closely related but different target site sequence specificities (Dehesh et al. 1992).

The SP8BF activity in nuclear extracts from petioles of sweet potato binds to DNA fragments carrying either the SP8a sequence or the SP8b sequence, with no

significant difference between the binding affinities for SP8a and SP8b sequences, judging from results of competition experiments (Ishiguro and Nakamura 1992). Although SPF1 binds to both of these sequences, the binding of SPF1 to oligo-SP8a is about 25 times more efficient than to oligo-SP8b (Fig. 4, B and C), suggesting that SPF1 binds much more strongly to the SP8a sequence than the SP8b sequence. It is possible that SPF1 synthesized *in vitro* is abnormally folded or modified or its ability to interact with other polypeptides may be altered relative to the native SPF1; this might explain the difference in the binding affinity for the SP8a and SP8b sequences. It is also possible that SP8BF activity in the nuclear extract is actually a mixture of several factors that show closely related but different binding site sequence specificities. Although SPF1 binds preferentially to the SP8a sequence, the other factor(s) may bind more selectively to the SP8b sequence. Nuclear extract from stems of tobacco also contains SP8BF activity (Ishiguro and Nakamura 1992). Our preliminary experiments using gel mobility shift assays of the tobacco nuclear extract with the oligo-SP8a probe, two or more distinguishable bands are observed (data not shown), suggesting that several nuclear factors in tobacco bind to the SP8a sequence. Although the SPF1 probe hybridized to either a single or a few restriction endonuclease fragments of the sweet potato DNA (data not shown), sweet potato may also contain additional factor(s) closely related to SPF1.

The SP8BF activity has been detected in nuclear extracts from petioles and tuberous roots of the sweet potato and from stems of tobacco (Ishiguro and Nakamura 1992). SPF1 mRNA was also detected not only in petioles but also in leaves, stems and tuberous roots of the sweet potato (Fig. 6A), suggesting that SPF1 is a ubiquitous factor. The level of SPF1 mRNA in petioles decreased after treatment with either 6% sucrose or 0.1% PGA (Fig. 6B). However, it is not clear at this moment whether SPF1 plays any negative regulatory role in the sucrose- or PGA-induced expression of genes coding for sporamin and β -amylase.

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